

Rho-associated kinase plays a role in rabbit urethral smooth muscle contraction, but not via enhanced myosin light chain phosphorylation

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Running title: Smooth muscle contraction without an increase in myosin phosphorylation

ABSTRACT

The involvement of Rho-associated kinase (ROK) in activation of rabbit urethral smooth muscle contraction was investigated by examining the effects of two structurally distinct inhibitors of ROK, Y27632 and H1152, on the contractile response to electric field stimulation (EFS), membrane depolarization with KCl, and α_1 -adrenoceptor stimulation with phenylephrine. Both compounds inhibited contractions elicited by all three stimuli. The protein kinase C inhibitor, GF109203X, on the other hand, had no effect. Urethral smooth muscle strips were analysed for phosphorylation of three potential direct or indirect substrates of ROK: (i) myosin regulatory light chains (LC₂₀) at S19; (ii) the myosin targeting subunit of myosin light chain phosphatase, MYPT1, at T697 and T855; and (iii) cofilin at S3. The following results were obtained: (i) under resting tension, LC₂₀ was phosphorylated to 0.65 ± 0.02 mol P_i/mol LC₂₀ ($n = 21$) at S19; (ii) LC₂₀ phosphorylation did not change in response to KCl or phenylephrine; (iii) ROK inhibition had no effect on LC₂₀ phosphorylation in the absence or presence of contractile stimuli; (iv) under resting conditions, MYPT1 was partially phosphorylated at T697 and

34T855 and cofilin at S3; (v) phosphorylation of MYPT1 and cofilin was unaffected by KCl or
35phenylephrine; and (vi) KCl- and phenylephrine-induced contraction-relaxation cycles did not correlate
36with actin polymerization-depolymerization. We conclude that ROK plays an important role in urethral
37smooth muscle contraction, but not via inhibition of MLCP or polymerization of actin.

38Key words: urethra, urinary continence, myosin light chain phosphatase, cofilin

39 40**INTRODUCTION**

41
42Lower urinary tract function is dependent on the concerted action of the smooth and striated muscles of
43the urinary bladder, urethra and periurethral region. Failure to store urine can lead to various forms of
44incontinence, which is a major health concern (26), but current therapies for incontinence have severe
45limitations (19). Further therapeutic development will depend on the identification of novel targets. The
46bladder and urethra work as a functional unit with a reciprocal relationship under normal conditions, i.e.,
47during the storage phase, the detrusor muscle of the bladder is relaxed while the urethra is contracted to
48allow gradual filling of the bladder with urine and prevent leakage. On the other hand, during voiding,
49the urethra relaxes and the detrusor contracts to facilitate emptying of the bladder (36). An isolated rat
50urethral preparation is “continent” in the absence of external neural input, but flow ensues when the
51smooth muscle is relaxed (29). Furthermore, stimulation of the skeletal muscle makes remarkably little
52difference to the ability of the contracted urethra to retain fluid. Conte et al (12) also found that
53paralyzing the striated muscle encircling the urethra of anesthetized rats with d-tubocurarine did not
54result in urine leakage. It would appear, therefore, that skeletal muscle is more important for resisting
55rapid pressure rises caused by coughing or laughing, for example, than for maintaining a constant
56urethral tone, which makes sense from an energetic standpoint. The smooth muscle cells of the urethra,
57therefore, play a critical role in continence by remaining in a contracted state most of the time, thereby
58retaining urine within the bladder. This smooth muscle tone can be modified by adrenergic and

59cholinergic nerve stimulation (4). Noradrenaline, released by adrenergic neurons, is the major excitatory
60transmitter in the rabbit urethra (3,13). At the appropriate time, the smooth muscle cells relax in
61response to inhibitory nerves, and detrusor smooth muscle contraction voids the bladder through a
62relaxed urethra. Nitric oxide, released by non-adrenergic, non-cholinergic neurons, is an important
63mediator of urethral smooth muscle relaxation (5,11).

64 Deficiencies in urethral closure can result in stress urinary incontinence. Treatment of this
65condition, which is based mainly on α_1 -adrenoceptor agonists, has been disappointing (6). In order to
66improve strategies for treatment of incontinence, it is essential to understand urethral function and
67regulation in greater detail.

68 Smooth muscle contraction and relaxation are regulated primarily by the phosphorylation and
69dephosphorylation of the 20-kDa regulatory light chains of myosin (LC₂₀) (2). Smooth muscle myosin II
70is a hexameric protein composed of two heavy chains (205 kDa) and two pairs of light chains (17 and 20
71kDa). Contraction is activated by an increase in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), whereupon
72 Ca^{2+} binds to calmodulin (CaM) and the $(\text{Ca}^{2+})_4$ -CaM complex activates myosin light chain kinase
73(MLCK). Activated MLCK phosphorylates LC₂₀ at S19, resulting in cross-bridge cycling of myosin
74along the actin filaments and contraction. Relaxation occurs when $[\text{Ca}^{2+}]_i$ returns to resting levels upon
75removal of the contractile stimulus, whereupon LC₂₀ is dephosphorylated by myosin light chain
76phosphatase (MLCP) and myosin dissociates from actin (18).

77 An important aspect of the regulation of smooth muscle contraction that has emerged in recent
78years concerns the phenomenon of Ca^{2+} sensitization, i.e., the ability of a variety of agonists to elicit a
79contractile response without an increase in $[\text{Ca}^{2+}]_i$ (33). Ca^{2+} sensitization involves agonist-induced
80activation of signaling pathways, primarily the RhoA/Rho-associated kinase (ROK) pathway, that
81terminate in the inhibition of MLCP. This shifts the balance between kinase and phosphatase in favor of

82MLCK so that a higher level of LC₂₀ phosphorylation (and force) is achieved at a given [Ca²⁺]_i. ROK
83mediates MLCP inhibition via phosphorylation of the myosin targeting subunit of the phosphatase
84(MYPT1) at T697 and/or T855 (rat numbering; NCBI accession number EDM16761) (16,31,39) or of
85the 17 kDa cytosolic protein, CPI-17, which becomes a potent inhibitor of MLCP when phosphorylated
86at T38 (27).

87 Studies regarding the role of LC₂₀ phosphorylation in urethral contractile physiology have been
88rather limited. Hypolite et al (21) reported that the rabbit urethra exhibits a low level of basal LC₂₀
89phosphorylation (12.8%). Modest increases in LC₂₀ phosphorylation, from 16% at rest to 28 % at half-
90maximal bethanecol-induced force, 29% at 80% maximal force and 27% at maximal force, were
91measured (21). A role for RhoA and ROK in urethral tone was indicated by the demonstration that
92inhibition of RhoA with *Clostridium difficile* toxin B or of ROK with Y27632 abolished porcine urethral
93tone without affecting cytosolic free Ca²⁺ concentration (28). ROK inhibition also inhibited the
94contractile response of rat urethral smooth muscle to phenylephrine, endothelin-1, α,β-methylene ATP
95and membrane depolarization, but had no significant effect on baseline tension (37).

96 The overall aim of this work, therefore, was to gain further insights into the molecular
97mechanisms involved in the regulation of urethral smooth muscle contraction. The following specific
98questions were addressed: (i) Is the contraction of urethral smooth muscle in response to electric field
99stimulation, membrane depolarization by KCl or α₁-adrenoceptor stimulation with phenylephrine
100attenuated by inhibition of ROK? (ii) Does the sustained contraction of urethral smooth muscle involve
101phosphorylation of LC₂₀? (iii) Does contraction of urethral smooth muscle correlate with the
102phosphorylation of MYPT1 at T697 and/or T855? (iv) Does the contraction-relaxation cycle of urethral
103smooth muscle correlate with actin polymerization-depolymerization?

106 MATERIALS AND METHODS

107

108 *Materials.* Antibodies: rabbit polyclonal anti-calponin was raised in-house against purified full-length
109 chicken gizzard calponin (42). Commercial antibodies were purchased from the following sources:
110 rabbit polyclonal anti-LC₂₀ (Santa Cruz Biotechnology) raised against the full-length human protein;
111 rabbit polyclonal anti-pS19-LC₂₀ (Rockland) raised against a synthetic phosphopeptide corresponding to
112 the region of the human protein containing pS19; rabbit polyclonal anti-CPI-17 (Upstate) raised against
113 full-length recombinant porcine CPI-17; rabbit polyclonal anti-actin (Cytoskeleton) raised against a
114 synthetic peptide corresponding to the C-terminal 11 residues of human actin (SGPSIVHRKCF); goat
115 polyclonal anti-SM-22 α (Novus Biologicals) raised against a synthetic peptide (MTGYGRPRQIIS)
116 corresponding to residues 189 - 200 of human SM-22 α ; rabbit polyclonal anti-pT697-MYPT1 (Upstate)
117 raised against a synthetic phosphopeptide corresponding to the region around pT697 of the human
118 protein; rabbit polyclonal anti-pT855-MYPT1 (Upstate) raised against a synthetic phosphopeptide
119 corresponding to the region around pT855 of the human protein; rabbit polyclonal anti-pS3-cofilin (Cell
120 Signaling Technology) raised against a synthetic phosphopeptide corresponding to human cofilin
121 containing phosphoserine at position 3; rabbit polyclonal anti-cofilin (Cell Signaling Technology) raised
122 against a synthetic peptide corresponding to human cofilin containing serine at position 3. N $_{\omega}$ -nitro-L-
123 arginine, atropine, phenylephrine and phorbol 12,13-dibutyrate were purchased from Sigma, Y27632
124 from BioMol International, H1152, GF109203X, wortmannin and calyculin-A from Calbiochem, and
125 microcystin-LR from Alexis Biochemicals. Molecular weight markers were purchased from Fermenta.

126 *Isolation of urethral tissue for tension measurements.* Male New Zealand white rabbits (3 - 4 kg)
127 were maintained and killed with a lethal injection of pentobarbitone according to the standards of the
128 Canadian Council on Animal Care and a protocol approved by the Animal Care Committee of the
129 Faculty of Medicine, University of Calgary, and in accordance with the European Union legislation and

130ethical standards. Male Sprague-Dawley rats (250 - 275 g) were maintained and killed by halothane
131inhalation and decapitation according to the standards of the Canadian Council on Animal Care and a
132protocol approved by the Animal Care Committee of the Faculty of Medicine, University of Calgary.
133The proximal 1 cm of the urethra was removed and placed in Krebs solution (120 mM NaCl, 5.9 mM
134KCl, 25 mM NaHCO₃, 5.5 mM glucose, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂) plus 100
135µM N_ω-nitro-L-arginine and 1 µM atropine with pH adjusted to 7.4 with 95% O₂/5% CO₂. The
136muscarinic antagonist atropine was included to block the effects of acetylcholine released from nerves,
137and N_ω-nitro-L-arginine to block nitric oxide effects. In separate experiments, we found that omission of
138these inhibitors had no statistically significant effect on LC₂₀ phosphorylation levels, as determined by
139paired Student's t test: 0.62 ± 0.11 mol P_i/mol LC₂₀ in their presence and 0.52 ± 0.10 mol P_i/mol LC₂₀ in
140their absence ($p > 0.05$, $n = 3$). Circularly-oriented strips (8 x 1 x 1 mm) of smooth muscle were
141dissected. For investigation of the effects of protein kinase inhibitors, muscle strips were placed in a
142water-jacketed organ bath maintained at 37 °C, and perfused with warmed Krebs solution bubbled with
14395% O₂/5% CO₂. Strips were adjusted to a tension of 0.5 g, the optimal tension for KCl-induced force
144development, and allowed to equilibrate for 60 min before experimentation began. During the period of
145equilibration of the tissue after mounting, it was necessary to stretch the tissue periodically to maintain
146resting tension at 0.5 g. Stable tension was always achieved within the 60-min equilibration period. Prior
147experiments indicated that a resting tension of 0.5 g for tissue strips of the dimensions used in this study
148gave a maximal contractile response to KCl. Contractions in response to electric field stimulation (EFS),
149KCl and phenylephrine were measured using Statham UC3 and Dynamometer UF1 transducers, with the
150outputs recorded on a Grass 7400 chart recorder. Tissues did not exhibit significant loss of maximal
151tension in response to repetitive stimuli (EFS, KCl or phenylephrine) over several hours. Field
152stimulation was applied via platinum ring electrodes mounted at either end of the tissue strip. Pulses of

1530.3 ms duration were delivered in trains at constant frequencies of 4 Hz from a Grass S48 stimulator at a
154nominal voltage of 50 V. Responses were blocked with 1 μ M tetrodotoxin, confirming that they were
155nerve mediated (38).

156 *Demembration (skinning) of urethral smooth muscle strips.* Urethral smooth muscle strips
157mounted on a force transducer at resting tension were incubated at 21 °C with 1% (v/v) Triton X-100 in
15830 mM TES, 50 mM KCl, 5 mM K₂EGTA, 150 mM sucrose, 0.5 mM dithioerythritol, pH 7.4 for 2 h. In
159establishing the optimal conditions for Triton skinning of rabbit urethral smooth muscle strips, the
160Triton X-100 concentration and time of treatment were varied. The contractile response of the Triton-
161skinned tissue to Ca²⁺ was comparable to that of the KCl-induced contraction of the intact tissue prior to
162Triton treatment when 1 % Triton X-100 was used for 2 h. Skinned tissues were then equilibrated with
1633.2 mM MgATP, 2 mM free MgCl₂, 0.5 mM NaN₃, 30 mM TES, 12 mM phosphocreatine, 15 units/ml
164creatine kinase, 1 μ M calmodulin, pH 6.9 with ionic strength adjusted to 150 mM with potassium
165propionate. Desired free Ca²⁺ levels were obtained by mixing stock solutions containing K₂EGTA and
166K₂CaEGTA (41).

167 *Protein extraction.* For investigation of protein phosphorylation, muscle strips were immersed in 10%
168trichloroacetic acid (TCA)/10 mM dithiothreitol (DTT) in acetone that had been pre-cooled on dry ice or
169wet ice, washed (3 x 1 min) with DTT/acetone, lyophilized overnight and the dried tissues cut into small
170pieces. SDS-PAGE sample buffer containing 0.1 M DTT (1 ml) was added. Tissue strips of comparable
171dimensions were extracted with identical volumes of SDS-gel sample buffer, and identical volumes of
172extract were loaded on gels for western blot analysis. The amount of extract loaded depended on the
173sensitivity of the individual antibodies utilized. The samples were heated at 95 °C for 10 min, rotated
174overnight in the cold room and stored at -20 °C until SDS-PAGE was performed.

175 *SDS-PAGE.* SDS-PAGE was carried out in 1.5-mm thick mini-gels (12.5% acrylamide in the

176running gel (15% for CPI-17 analysis) with a 5% acrylamide stacking gel) at 200 V for 45 min in a Mini
177Protean 3 Cell (Bio-Rad). Following electrophoresis, gels were either stained with Coomassie Brilliant
178Blue or equilibrated at room temperature for 1 h with Transblot Buffer (25 mM Tris-HCl, pH 7.5, 192
179mM glycine, 20% methanol, 0.1% SDS for MYPT1; 10 mM CAPS, pH 11, 10% methanol for CPI-17;
18025 mM Tris-HCl, pH 7.5, 192 mM glycine, 20% methanol for the rest) prior to western blotting.

181 *Western blotting.* Proteins separated by SDS-PAGE were transblotted to nitrocellulose (0.2 μ m; Bio-
182Rad) at 100 V for 1 h at 4 °C in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). After
183blotting, the nitrocellulose membrane was washed (3 x 5 min) in PBS (0.137 M NaCl, 2.68 mM KCl, 10
184mM Na₂HPO₄, 1.76 mM KH₂PO₄), incubated in 0.5% glutaraldehyde in PBS for 15 min to fix proteins
185on the membrane and washed (3 x 5 min) with Tris-buffered saline containing Tween 20 (TBST: 20 mM
186Tris-HCl, pH 7.5, 137 mM NaCl, 3 mM KCl, 0.05% Tween-20). The membrane was then blocked with
1875% non-fat dried milk in TBST for 1 h, and incubated overnight at 4 °C with 1% non-fat dried milk in
188TBST containing the appropriate primary antibody. Membranes were washed (4 x 5 min) in TBST,
189incubated with anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution
190in 1% dry milk in TBST; Chemicon) for 1 - 2 h and washed (4 x 5 min) with TBST and (1 x 5 min) with
191TBS before chemiluminescence signal detection using the Super-Signal West Femto reagent (Pierce).
192The emitted light was detected and quantified with a chemiluminescence imaging analyzer
193(LAS3000mini; Fujifilm) and images were analysed with MultiGauge v3.0 software (Fujifilm).

194 *Phosphate affinity SDS-PAGE using acrylamide-pendant phosphate-binding tag.* Unphosphorylated
195and phosphorylated forms of LC₂₀ were separated by Phos-tag SDS-PAGE (23,35). Muscle protein
196samples stored in SDS-PAGE sample buffer at -20 °C were electrophoresed at 30 mA/gel for 60 min in
197mini-gels in which 0.05 mM Phos-tag acrylamide (NARD Institute, Japan) and 0.1 mM MnCl₂ were
198incorporated into the running gel. In control experiments, 1 mM EDTA replaced 0.1 mM MnCl₂. After

199electrophoresis, gels were soaked in Transblot Buffer (25 mM Tris-HCl, pH 7.5, 192 glycine, 10%
200methanol) containing 2 mM EDTA for 15 min prior to equilibration in Transblot Buffer for 15 min.
201Proteins were transferred to PVDF (Roche) at 27 V overnight at 4 °C and fixed with 0.5%
202glutaraldehyde prior to blocking, etc. as for the normal western blotting procedure described above.

203 *Actin polymerization assay.* Actin polymerization was measured using the G-actin/F-actin *in vivo*
204Assay Kit (Cytoskeleton). Rabbit urethral smooth muscle strips, mounted on a force transducer and
205equilibrated with Krebs solution at 21 °C, were treated as described in the appropriate figure legends,
206homogenized in F-actin stabilization buffer (1 ml) with a Tissue Tearor Model 985-370 Type 2 (Biospec
207Products) at 21 °C and centrifuged in a bench-top centrifuge at 2,000 rpm for 5 min. Supernatants were
208centrifuged at 100,000 x g for 1 h at 21 °C. The high-speed supernatant was carefully removed and 50 µl
209of 5X SDS-gel sample buffer added. The high-speed pellet was re-suspended in 250 µl of 1x SDS-gel
210sample buffer. Samples were heated at 95 °C for 5 min and stored at -20 °C prior to SDS-PAGE and
211western blotting.

212 *Statistical analysis.* Statistical analyses were performed with SigmaPlot and Prism. Results are
213expressed as the means ± S.E.M. Values of *n* indicate the numbers of animals used; several tissue strips
214were used from each animal. Statistically significant differences were identified by Student's *t* test or
215one-way ANOVA with Dunnett's post-hoc test as indicated.

216

217RESULTS

218 *Effects of ROK inhibition on urethral smooth muscle contraction.* The effects of ROK inhibition on the
219contractile response of rabbit urethral smooth muscle to EFS, membrane depolarization with KCl, and
220α₁-adrenoceptor stimulation by phenylephrine were investigated at 37 °C. Following dissection,
221mounting and equilibration of the tissue, several control contraction-relaxation cycles were recorded.

222The tissue was then incubated with ROK inhibitor (Y27632 or H1152) for 15 min prior to two additional
223contraction-relaxation cycles in the continued presence of inhibitor. The ROK inhibitor was then washed
224out and two control contraction-relaxation cycles recorded again. Representative data showing the
225effects of H1152 on these contractile responses are shown in Fig. 1*A* with cumulative quantitative data
226for both inhibitors in Fig. 1*B*. The contractile responses to EFS, KCl or phenylephrine were all markedly
227inhibited by both ROK inhibitors (Fig. 1*A,B*). Furthermore, inhibition of ROK during steady-state force
228maintenance in the presence of phenylephrine elicited relaxation (Fig. 1*C*). The general protein kinase C
229(PKC) inhibitor, GF109203X, on the other hand, had no significant effect on the contractile response of
230the urethra to any of the stimuli (Supplementary Fig. 1). The efficacy of the PKC inhibitor was verified
231by its ability to block the contractile response of the rabbit urethra to 0.5 μ M phorbol 12,13-dibutyrate
232(PdBu): steady-state force in response to 0.5 μ M PdBu (0.47 ± 0.13 g) was reduced to 0.20 ± 0.09 g ($n =$
2336; $p < 0.01$) following pre-incubation with 2 μ M GF109203X. It is noteworthy that the contractile
234response to PdBu was very slow and reached a steady-state level of force that was much less than that
235induced by KCl or phenylephrine.

236 In order to pursue the mechanism underlying the contractile responses and the effects of ROK
237inhibition shown in Fig. 1, it was necessary to use an experimental system that is amenable to rapid
238quenching for biochemical analysis. For this purpose, urethral muscle strips were mounted in a 1-ml
239cuvette at room temperature and the effects of ROK inhibition on phenylephrine- and KCl-induced
240contraction examined. Fig. 2*A* shows the inhibition of phenylephrine-induced contraction by H1152 (1
241 μ M): the mean maximal tension in response to phenylephrine in the presence of H1152 was 56.8% of
242control, compared to 118% of control following washout of the ROK inhibitor. The concentration
243dependence of H1152-induced relaxation of tissue pre-contracted with phenylephrine is shown in Fig.
2442*B*. Additional experiments in which longer time intervals were used between successive additions of

245H1152 indicated that the concentration required for half-maximal relaxation was 0.3 μ M H1152 (Fig.
2462C). H1152 had a similar inhibitory effect on KCl-induced contraction under these conditions (data not
247shown).

248 These results indicate that contraction of rabbit urethral smooth muscle evoked by EFS,
249membrane depolarization or α_1 -adrenoceptor activation involves activation of ROK, but not PKC, and
250suggest that contraction of the urethra may involve a significant Ca^{2+} sensitization. Activation of ROK
251has been implicated in the inhibition of MLCP through the phosphorylation of MYPT1 at T697 and/or
252T855 (16,31,39). ROK is also known to phosphorylate CPI-17 at T38, which converts it into a potent
253inhibitor of MLCP (27). Inhibition of MLCP activity would shift the kinase-phosphatase activity balance
254in favour of MLCK and therefore increase LC_{20} phosphorylation and force.

255 *LC_{20} phosphorylation.* To initiate the investigation of the mechanism whereby ROK regulates urethral
256smooth muscle contraction, we measured LC_{20} phosphorylation levels in extracts of tissues quenched at
257rest and at the peak of contraction induced by phenylephrine or KCl. Phosphorylated and
258unphosphorylated LC_{20} were separated by Phos-tag SDS-PAGE and detected by western blotting with
259an antibody that recognizes both forms of the light chain (Fig. 3A): LC_{20} was phosphorylated to ~ 0.6
260 $\text{mol P}_i/\text{mol LC}_{20}$ at resting tension (lanes 5 and 6). Western blotting with a phosphospecific antibody that
261recognizes only LC_{20} phosphorylated at S19, the MLCK site (Fig. 3B), and Phos-tag SDS-PAGE in the
262presence of EDTA to chelate Mn^{2+} ions (Fig. 3C), confirmed the identities of the bands as labelled.
263Thus, only the slower migrating band was recognized by the phosphospecific antibody (Fig. 3B) and,
264when Mn^{2+} ions were chelated with EDTA, LC_{20} migrated as a single band with the mobility of
265unphosphorylated LC_{20} observed in the presence of Mn^{2+} (Fig. 3C). Surprisingly, treatment with
266phenylephrine (Fig. 3A, lanes 1 and 2) or KCl (Fig. 3A, lanes 8 and 9) did not increase the level of LC_{20}
267phosphorylation. The cumulative quantitative data in Table 1 confirm this conclusion. Also shown in

268 Fig. 3A (lanes 3 and 4) is LC₂₀ mono- and diphosphorylation (S19 and T18) in rabbit urethral smooth
269 muscle strips contracted in response to the phosphatase inhibitor, calyculin-A. No LC₂₀
270 diphosphorylation was detected in response to phenylephrine or KCl (Fig. 3B). Pre-incubation with
271 H1152 had no effect on LC₂₀ phosphorylation in the presence of KCl or phenylephrine (Fig. 3D and
272 Table 1).

273 The possibility arose that because LC₂₀ phosphorylation was quantified after steady-state force
274 was achieved, a transient increase in LC₂₀ phosphorylation correlating with force development may have
275 been missed. Therefore, the time courses of LC₂₀ phosphorylation in response to treatment with KCl and
276 phenylephrine were investigated. Fig. 4 shows that there was, in fact, no change in LC₂₀ phosphorylation
277 throughout the time course of KCl- or phenylephrine-induced contraction.

278 *MYPT1 phosphorylation.* Since MYPT1 is a well known substrate of ROK in various smooth muscles,
279 we examined the phosphorylation of MYPT1 at the two ROK sites by western blotting with
280 phosphospecific antibodies. Both sites were phosphorylated at resting tension and neither KCl nor
281 phenylephrine changed the level of phosphorylation at either site at the peak of the contractile response
282 (Fig. 5 and Table 2). Analysis of the time course of phosphorylation revealed that MYPT1
283 phosphorylation at T697 and T855 did not change during the contraction elicited by either KCl (Fig. 6A
284 and C) or phenylephrine (Fig. 6B and C). In order to determine whether T697 and T855 were
285 stoichiometrically or only partially phosphorylated, MYPT1 phosphorylation in intact rabbit urethral
286 strips treated with KCl was compared with Triton-skinned tissues treated with the phosphatase inhibitor
287 microcystin. Resting levels of MYPT1 phosphorylation at T697 and T855 were unaffected by KCl
288 treatment of intact tissues, whereas substantial increases in phosphorylation at both sites occurred upon
289 treatment of the demembrated tissue with microcystin (Fig. 7). Densitometric analysis of the western
290 blots gave a ratio of KCl:control signals (normalized to calponin) of 1.03 ± 0.13 and 0.79 ± 0.21 (mean

291± SEM, $n = 3$) for T697 and T855, respectively, and a ratio of microcystin:control of 2.03 ± 0.08 and 2928.78 ± 1.00 (mean ± SEM, $n = 3$) for T697 and T855, respectively.

293 *CPI-17*. Although CPI-17 is well established as a PKC substrate (24,27), it has also been implicated as
294a ROK substrate, at least *in vitro* (15,25). Phosphorylation by both kinases occurs at T38 and renders
295CPI-17 a potent MLCP inhibitor. Therefore, we investigated the expression of CPI-17 in the rabbit
296urethra. Consistent with previous findings with non-vascular smooth muscles (43), the level of CPI-17 in
297the urethra is considerably less than in vascular smooth muscles and comparable to the level in bladder
298(Supplementary Fig. 2). Given the low tissue content of CPI-17, the fact that LC₂₀ phosphorylation was
299unaffected by ROK inhibition (Fig. 3D and Table 1) and the lack of effect of PKC inhibition on urethral
300contractility (Supplementary Fig. 1), we decided not to pursue the analysis of CPI-17 phosphorylation.

301 *Cofilin phosphorylation*. LIM kinase (10) has also been shown to be a ROK substrate, and
302phosphorylation at T508 within the activation loop activates this kinase (32). Activated LIM kinase in
303turn phosphorylates the actin capping and severing protein, cofilin, at S3 (7,44). In the unphosphorylated
304state, cofilin binds to and severs actin filaments and this effect is alleviated upon phosphorylation at S3
305(1). We examined the time course of cofilin phosphorylation in rabbit urethral smooth muscle in
306response to KCl and phenylephrine by western blotting with a phosphospecific antibody that recognizes
307cofilin only when phosphorylated at S3. As shown in Fig. 8, cofilin is phosphorylated at S3 in the
308unstimulated tissue and its phosphorylation level does not change significantly during the time course of
309contraction in response to either KCl or phenylephrine.

310 *Actin polymerization*. We also measured actin polymerization during the time course of the contractile
311response to KCl and phenylephrine. Urethral tissue strips were homogenized in F-actin stabilization
312solution at the times indicated in Fig. 4A and B, and F- and G-actin were separated by high-speed
313centrifugation. Fig. 9A demonstrates that SM-22, a 22 kDa smooth muscle-specific protein (30), was

314 recovered exclusively in the high-speed supernatant and, therefore, provides a suitable protein for
315 normalization of loading levels. The high-speed supernatant, containing all the G-actin, was then
316 analysed by western blotting with anti-actin and loading levels were normalized to SM-22 (Fig. 9B). If
317 KCl and phenylephrine induce actin polymerization that is required for force production in the urethra,
318 we would anticipate that the G-actin content would decline rapidly in response to both stimuli and would
319 increase again during relaxation upon washout. This was not found to be the case (Fig. 9B).

320 *Comparison of LC₂₀ phosphorylation in rabbit urethra, bladder and aorta.* The very high level of
321 basal phosphorylation of LC₂₀ in the rabbit urethra at resting tension (0.65 mol P_i/mol LC₂₀) was
322 unexpected, as was the absence of an increase in response to membrane depolarization or α_1 -
323 adrenoceptor activation (Table 1). Therefore, we investigated whether the same was true for another
324 phasic urogenital smooth muscle (bladder) and a tonic vascular smooth muscle (aorta). As for the
325 urethra, the basal level of LC₂₀ phosphorylation in bladder smooth muscle was high and did not
326 significantly change in response to KCl or phenylephrine (Table 3). On the other hand, the basal level of
327 LC₂₀ phosphorylation in the aorta was lower and did increase in response to both KCl and phenylephrine
328 (Table 3).

329 *LC₂₀ phosphorylation in rat urethra and bladder.* The role of LC₂₀ phosphorylation in contraction of
330 urethra and bladder smooth muscles was also investigated in rat tissues. In contrast to the situation with
331 rabbit tissues, LC₂₀ phosphorylation in rat urethra and bladder was relatively low at resting tension and
332 increased in response to both KCl and phenylephrine (Table 4).

333 *Comparison of methods for quenching of tissues prior to LC₂₀ phosphorylation analysis.* During the
334 course of this work, we discovered that the method for quenching of rabbit urethral smooth muscle strips
335 for biochemical analysis was crucial. This is often achieved by immersing the tissue in
336 TCA/acetone/DTT on dry ice or clamping the tissue between liquid nitrogen-cooled tongs, immersing it

337in TCA/acetone/DTT on dry ice and slowly warming the tissue to room temperature in
338TCA/acetone/DTT. However, we found that these procedures resulted in very low levels of LC₂₀
339phosphorylation (Table 5). On the other hand, immersing the tissue in TCA/acetone/DTT on wet ice
340gave much higher and reproducible phosphorylation stoichiometry (Table 1). We also quenched the
341tissues by immersion in liquid nitrogen, followed by transfer directly to boiling SDS-gel sample buffer.
342After incubation at 95 °C for 10 min, samples were rotated overnight at 4 °C prior to Phos-tag SDS-
343PAGE. As shown in Supplementary Figure 3 and Supplementary Table 1, very low levels of LC₂₀
344phosphorylation stoichiometry were measured under these conditions. In addition, rabbit urethral
345smooth muscle strips were clamped between liquid nitrogen-cooled tongs at rest and at the plateau of
346KCl- or phenylephrine-induced contractions and then treated in one of the following ways: (i) tissues
347were immersed in TCA/acetone/DTT on wet ice; (ii) tissues were immersed in TCA/acetone/DTT on
348dry ice; or (iii) tissues were immersed in liquid N₂. Tissues from (i) and (ii) were then lyophilized
349overnight after washing with acetone/DTT. All three sets of tissues were then immersed in boiling SDS-
350gel sample buffer, maintained at 95 °C for 10 min, and rotated at 4 °C overnight prior to Phos-tag SDS-
351PAGE to quantify LC₂₀ phosphorylation levels. The results confirmed a high level of resting LC₂₀
352phosphorylation when tissues freeze-clamped with liquid N₂-cooled tongs were immersed in
353TCA/acetone/DTT on wet ice (Supplementary Table 2). On the other hand, much lower levels of LC₂₀
354phosphorylation were measured when tissues freeze-clamped with liquid N₂-cooled tongs were
355immersed in TCA/acetone/DTT on dry ice. Even lower levels of LC₂₀ phosphorylation were measured
356when tissues freeze-clamped with liquid N₂-cooled tongs were immersed in liquid N₂.

357 *The effects of removal of extracellular Ca²⁺ and pre-incubation with wortmannin on rabbit urethral*
358*smooth muscle contraction and LC₂₀ phosphorylation.* Finally, to identify the kinase responsible for the
359high level of LC₂₀ phosphorylation in the rabbit urethra under resting conditions, the effect on LC₂₀

360phosphorylation of removal of extracellular Ca^{2+} and pre-incubation with wortmannin (10 μM), which at
361this concentration is a MLCK inhibitor, was investigated. Incubation of urethral tissue strips in Ca^{2+} -free
362Krebs solution containing 10 mM EGTA for 30 min resulted in a marked decrease in LC_{20}
363phosphorylation and resting tension (Table 6). Treatment with KCl (80 mM) or phenylephrine (10 μM)
364after pre-incubation of tissue strips in Ca^{2+} -free Krebs solution containing 10 mM EGTA for 30 min
365failed to elicit a contractile response or an increase in LC_{20} phosphorylation (Table 6). Preincubation of
366tissue strips with wortmannin (10 μM) in Krebs solution also reduced the resting level of LC_{20}
367phosphorylation and reduced resting tension by over 50% (Table 6). Addition of KCl (80 mM) after
368incubation with wortmannin for 30 min failed to elicit a contractile response or an increase in LC_{20}
369phosphorylation (Table 6).

370

371 **DISCUSSION**

372 The most interesting findings from this study were that the urethra and bladder of the rabbit had a
373high level of LC_{20} phosphorylation at resting tension, and that membrane depolarization and α_1 -
374adrenoceptor stimulation did not increase the level of LC_{20} phosphorylation (Figs. 3 and 4 and Table 1),
375in spite of inducing robust contractile responses (Figs. 1 and 2). Vascular smooth muscle of the rabbit
376(aorta), on the other hand, behaved as expected, i.e., LC_{20} phosphorylation increased in response to
377membrane depolarization and α_1 -adrenoceptor stimulation (Table 3). Rat urethra and bladder, however,
378had a low level of LC_{20} phosphorylation at rest, which increased in response to membrane depolarization
379and α_1 -adrenoceptor stimulation, indicating species specificity (Table 4). It will be important in the
380future to determine whether human urethral and bladder smooth muscles behave like the rabbit or rat
381counterparts.

382 Another key finding from this study was that the contractile responses to electric field stimulation,

383 KCl and phenylephrine were all potently inhibited by Y27632 and H1152, two structurally unrelated
384 inhibitors of ROK (Figs. 1 and 2). It is important to note that, although highly selective, these
385 compounds can inhibit other kinases such as PRK2, AMPK and, to a lesser degree, PDK11 (9). PRK2
386 and PDK11 have not been implicated in the regulation of smooth muscle contraction and AMPK has
387 been shown to phosphorylate and inactivate smooth muscle MLCK (20). Given our observation that
388 Y27632 and H1152 had no effect on LC₂₀ phosphorylation (Fig. 3D and Table 1), it is unlikely that the
389 inhibitory effects of Y27632 and H1152 on contraction involve inhibition of AMPK.

390 The RhoA/ROK pathway has previously been implicated in agonist-induced contraction of the rat
391 urethra (37) and spontaneous tone in female porcine urethral smooth muscle (28). Extensive studies of
392 various smooth muscles have implicated the RhoA/ROK pathway in Ca²⁺ sensitization, i.e., an increase
393 in force at a given [Ca²⁺]_i. This pathway results in inhibition of MLCP via phosphorylation of the
394 myosin targeting subunit of MLCP (MYPT1) and/or CPI-17 by ROK, which results in increased LC₂₀
395 phosphorylation and contraction (33). Since ROK inhibition did not alter LC₂₀ phosphorylation in rabbit
396 urethral smooth muscle (Fig. 3 and Table 1), we concluded that MLCP inhibition is not involved in the
397 contractile response to KCl or phenylephrine. This conclusion was supported by the fact that MYPT1
398 phosphorylation at the two inhibitory ROK sites did not increase in response to either stimulus (Figs. 5
399 and 6 and Table 2), although phosphorylation could be greatly enhanced by treatment with the
400 phosphatase inhibitor, microcystin (Fig. 7), indicating that resting phosphorylation at T697 and T855
401 was sub-stoichiometric. We did not pursue the phosphorylation of CPI-17 since tissue levels of this
402 protein were very low in comparison to vascular smooth muscles (Supplementary Fig. 2), consistent
403 with reports in the literature that CPI-17 is most highly expressed in tonic vascular smooth muscles and
404 least in phasic visceral smooth muscles (43), and PKC, which is known to phosphorylate CPI-17 at T38,
405 is not involved in rabbit urethral smooth muscle contraction in response to electric field stimulation,

406membrane depolarization or α_1 -adrenoceptor stimulation (Supplementary Fig. 1). Furthermore, the fact
407that ROK inhibition had no effect on LC₂₀ phosphorylation supports the conclusion that CPI-17 does not
408have a role in the contractile responses observed.

409 Another ROK substrate that may play a role in regulation of contractility is LIM kinase, which
410contains two LIM domains, zinc finger domains originally identified in the proteins Lin11, Isl-1 and
411Mec-3 (8). This kinase is activated by ROK-catalysed phosphorylation within the activation loop. The
412activated LIM kinase phosphorylates the actin capping and severing protein cofilin at S3, whereupon it
413loses the ability to bind to actin and sever actin filaments (10). Actin polymerization has been implicated
414in smooth muscle contraction, and dephosphorylation of cofilin at S3 favors actin polymerization by
415increasing the availability of barbed ends of actin filaments (17). This would predict that
416dephosphorylation of cofilin, and not ROK/LIM kinase-mediated phosphorylation at S3 would occur in
417response to contractile stimulation, and a decrease in phosphocofilin has been demonstrated in canine
418tracheal smooth muscle treated with acetylcholine or KCl, which is associated with actin polymerization
419and contraction (45). Nevertheless, we investigated cofilin phosphorylation in rabbit urethral smooth
420muscle. While cofilin exhibited a basal level of S3 phosphorylation under resting tension, there was no
421change in cofilin phosphorylation in response to membrane depolarization or α_1 -adrenoceptor
422stimulation (Fig. 8). Furthermore, there was no change in G-actin content in response to KCl or
423phenylephrine (Fig. 9). We concluded, therefore, that the ROK/LIM kinase/cofilin pathway is not
424responsible for membrane depolarization- or α_1 -adrenoceptor stimulation-induced contraction of rabbit
425urethral smooth muscle.

426 The phosphatase inhibitor calyculin-A induced diphosphorylation of LC₂₀ (Fig. 3A and B) and
427contraction of intact urethral smooth muscle (data not shown). We have shown previously that LC₂₀
428diphosphorylation occurs at S19 and T18 in vascular smooth muscle, and is catalysed by integrin-linked

429kinase, not MLCK (14,40). We did not observe any LC₂₀ diphosphorylation in urethral smooth muscle
430under resting conditions or in response to KCl or phenylephrine (e.g., Fig. 3) and conclude that ILK is
431not responsible for the high level of LC₂₀ phosphorylation observed at rest or in the presence of these
432contractile stimuli. ROK has been shown to phosphorylate LC₂₀ directly *in vitro*, although this does not
433appear to occur *in situ* (34). The fact that ROK inhibition by H1152 had no effect on LC₂₀
434phosphorylation under resting conditions or in the presence of KCl or phenylephrine (Fig. 3D and Table
4351) indicates that ROK is not responsible for the observed phosphorylation of LC₂₀. At resting tension,
436LC₂₀ phosphorylation was almost abolished by removal of extracellular Ca²⁺ or by addition of
437wortmannin (MLCK inhibitor) in the presence of 2.5 mM extracellular Ca²⁺ (Table 6). Furthermore, KCl
438and phenylephrine failed to elicit a contractile response in the absence of extracellular Ca²⁺ or in the
439presence of wortmannin at 2.5 mM extracellular Ca²⁺ (Table 6). These results indicate that MLCK is the
440kinase responsible for LC₂₀ phosphorylation in the rabbit urethra at rest and in the presence of KCl and
441phenylephrine.

442 The method used to quench the urethral tissue strips for biochemical analysis proved crucial. If tissues
443were immersed in TCA/acetone/DTT on dry ice, or clamped between liquid nitrogen-cooled tongs prior
444to immersion in TCA/acetone/DTT on dry ice and gradual warming to room temperature, the levels of
445LC₂₀ phosphorylation were consistently very low at rest and did not change in response to KCl or
446phenylephrine (Table 5). On the other hand, if tissues were immersed in TCA/acetone/DTT on wet ice,
447the levels of LC₂₀ phosphorylation were consistently much higher at rest, and again did not change in
448response to KCl or phenylephrine (Table 1). These results suggest that the use of TCA/acetone/DTT on
449dry ice does not rapidly quench cellular biochemical reactions so that LC₂₀ is dephosphorylated by
450MLCP that remains active under these conditions. If the tissue was freeze-clamped between liquid
451nitrogen-cooled tongs, and subsequently immersed in TCA/acetone/DTT on wet ice or dry ice, or in

liquid nitrogen, differences in LC₂₀ phosphorylation levels were again observed (Table 5 and Supplementary Table 2). In the case of immersion in wet ice-cooled TCA/acetone/DTT, resting LC₂₀ stoichiometry was 0.36 mol P_i/mol LC₂₀, which did not change in response to contractile stimuli (Supplementary Table 2). On the other hand, very low levels of LC₂₀ phosphorylation were measured in the cases of tissues that had been freeze-clamped between liquid nitrogen-cooled tongs and then immersed in dry-ice-cooled TCA/acetone/DTT or liquid nitrogen. Furthermore, for tissues that were immersed directly in liquid nitrogen and then transferred to boiling SDS-gel sample buffer, the measured stoichiometry of LC₂₀ phosphorylation was again very low (Supplementary Fig. 3 and Supplementary Table 1), consistent with a very high tissue phosphatase activity. We interpret these results as follows: freeze-clamping the tissue between liquid nitrogen-cooled tongs, or plunging the tissue in liquid nitrogen or dry ice-cooled TCA/acetone/DTT dramatically reduces the phosphatase activity, but as the tissue warms up in SDS-gel sample buffer or TCA/acetone/DTT the phosphatase becomes active again and dephosphorylates LC₂₀ before a sufficiently high temperature is reached to denature the phosphatase. It appears unlikely that immersion of tissue in wet ice-cooled TCA/acetone/DTT would lead to activation of MLCK since the kinase requires Ca²⁺, Mg²⁺ and ATP for activity and none are present in the quench solution. Our observation that immersion of unstimulated rat tissues in wet ice-cooled TCA/acetone/DTT gave the expected low basal levels of LC₂₀ phosphorylation also argues against activation of MLCK under these quenching conditions. It is also clear from the results presented that any manipulation involving rapid freezing of the rabbit urethra results in low levels of LC₂₀ phosphorylation under both resting and stimulated conditions. In particular, it is apparent from Supplementary Table 2 that freeze-clamping the tissue with liquid nitrogen-cooled tongs followed by immersion in wet ice-cooled TCA/acetone/DTT yields lower and more variable LC₂₀ phosphorylation stoichiometry (0.36 ± 0.07 mol P_i/mol LC₂₀) than direct immersion in wet ice-cooled

475TCA/acetone/DTT (0.65 ± 0.02 mol P_i /mol LC_{20}), consistent with phosphatase activity
476dephosphorylating LC_{20} during sample work-up. Furthermore, we compared the basal level of LC_{20}
477phosphorylation at 0.5 g resting tension with that when no tension was applied to the tissue, and found
478no statistically significant difference by Student's t-test ($p > 0.05$): 0.66 ± 0.03 ($n = 18$) and 0.71 ± 0.11
479($n = 10$) mol P_i /mol LC_{20} , respectively. Also, if tissue at zero tension was quenched with dry ice-cooled
480TCA/acetone/DTT, low levels of LC_{20} phosphorylation were measured (0.11 ± 0.05 mol P_i /mol LC_{20} ; n
481= 6). Caution must, therefore, be exercised when choosing a method of tissue quenching for
482quantification of LC_{20} phosphorylation in rabbit tissues.

483 Future studies will be directed towards identification of ROK substrates in rabbit urethra with a
484view to defining the mechanism of activation of contraction without an increase in LC_{20}
485phosphorylation.

486

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492

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496

497DISCLOSURES

498 No conflicts of interest are declared by the authors.

499

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Table 1 LC₂₀ phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine, and the effect of ROK inhibition

Conditions	mol P _i /mol LC ₂₀
Control	0.65 ± 0.02 (21)
KCl	0.63 ± 0.03 (22)
Phenylephrine	0.62 ± 0.03 (19)
H1152	0.55 ± 0.05 (7)
KCl + H1152	0.56 ± 0.03 (6)
Phenylephrine + H1152	0.57 ± 0.06 (7)

Rabbit urethral smooth muscle strips were treated with KCl, phenylephrine or vehicle with or without pre-incubation with the ROK inhibitor H1152 (see legend to Fig. 2). Tissues were immersed in TCA/acetone/DTT on wet ice at the peak of contraction, and LC₂₀ phosphorylation was analysed by Phos-tag SDS-PAGE. Values indicate the mean ± SEM (*n* values given in parentheses). No significant differences from control were observed by one-way ANOVA with Dunnett's post-hoc test (*p* > 0.05).

Table 2 MYPT1 phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine

Conditions	pT697	pT855
KCl	0.86 ± 0.09 (7)	0.88 ± 0.118 (7)
Phenylephrine	1.25 ± 0.21 (9)	1.38 ± 0.14 (9)

Rabbit urethral smooth muscle strips were treated with KCl, phenylephrine, or vehicle (see legend to Fig. 2). Tissues were immersed in TCA/acetone/DTT on wet ice at the peak of contraction, and MYPT1 phosphorylation at T697 and T855 was analysed by western blotting with phosphospecific antibodies. Values indicate signal intensities relative to control after normalization of loading levels ± SEM with *n* values in parentheses. No significant differences from control (set at a value of 1) were observed by Student's *t* test (*p* > 0.05).

Table 3 LC₂₀ phosphorylation in rabbit urethral, bladder and aortic smooth muscles treated with KCL or phenylephrine

Conditions	Urethra (mol P _i /mol LC ₂₀)	Bladder (mol P _i /mol LC ₂₀)	Aorta (mol P _i /mol LC ₂₀)
Control	0.67 ± 0.07	0.63 ± 0.06	0.45 ± 0.05
KCl	0.70 ± 0.03	0.67 ± 0.01	0.59 ± 0.05*
Phenylephrine	0.70 ± 0.02	0.59 ± 0.06	0.65 ± 0.04*

Rabbit urethral, bladder and aortic smooth muscle strips were treated with KCl, phenylephrine, or vehicle (see legend to Fig. 2). Tissues were immersed in TCA/acetone/DTT on wet ice at the peak of contraction, and LC₂₀ phosphorylation was analysed by Phos-tag SDS-PAGE. Values indicate the mean ± SEM (*n* = 6 for urethra, 3 for bladder and 5 for aorta). Statistically significant differences from control are indicated with asterisks (**p* < 0.05) as determined by one-way ANOVA with Dunnett's post-hoc test.

643 *Table 4 LC₂₀ phosphorylation in rat urethral and bladder smooth muscles treated with KCl or*
644 *phenylephrine*

645

Conditions	Urethra (mol P _i /mol LC ₂₀)	Bladder (mol P _i /mol LC ₂₀)
Control	0.13 ± 0.05	0.21 ± 0.06
KCl	0.37± 0.07*	0.43 ± 0.06*
Phenylephrine	0.41 ± 0.04**	0.45 ± 0.06*

646

647Rat urethral and bladder smooth muscle strips were treated with KCl, phenylephrine, or vehicle (see
648legend to Fig. 2). Tissues were immersed in TCA/acetone/DTT on wet ice at the peak of contraction,
649and LC₂₀ phosphorylation was analysed by Phos-tag SDS-PAGE. Values indicate the mean ± SEM (*n* =
6504). Statistically significant differences from control are indicated with asterisks (**p* < 0.5; ***p* < 0.01),
651as determined by one-way ANOVA with Dunnett’s post-hoc test.

652

Table 5 LC₂₀ phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine, and quenched in TCA/acetone/DTT on dry ice or clamped between liquid nitrogen-cooled tongs

Conditions	Treatment (i) (mol P _i /mol LC ₂₀)	Treatment (ii) (mol P _i /mol LC ₂₀)
Control	0.05 ± 0.01 (14)	0.04 ± 0.01 (3)
KCl	0.12 ± 0.03 (10)	0.07 ± 0.02 (3)
Phenylephrine	0.07 ± 0.02 (11)	0.04 ± 0.01 (3)

Rabbit urethral smooth muscle strips were treated with KCl, phenylephrine or vehicle (see legend to Fig. 592). (i) Tissues at the peak of contraction were immersed in TCA/acetone/DTT on dry ice, and then washed (3 x 1 ml) in acetone/DTT, the liquid poured off and the tissue lyophilized; or (ii) tissues at the peak of contraction were quick frozen by clamping between liquid nitrogen cooled tongs, immersed in TCA/acetone/DTT on dry ice, slowly (1 h) warmed to room temperature, washed (3 x 1 ml) in acetone/DTT, the liquid poured off and the tissue was frozen on dry ice and lyophilized. LC₂₀ phosphorylation was analysed by Phos-tag SDS-PAGE. Values indicate the mean ± SEM (*n* values in parentheses). No statistically significant differences from control were detected by one-way ANOVA with Dunnett's post-hoc test (*p* > 0.05).

Table 6 The effects of removal of extracellular Ca^{2+} and pre-incubation with wortmannin on LC_{20} phosphorylation and contraction in rabbit urethral smooth muscle

Conditions	mol P_i /mol LC_{20}	Contractile response
Control	0.70 ± 0.02	None
0 Ca^{2+}	$0.13 \pm 0.08^{**}$	Relaxation ($43.8 \pm 5.8\%$)
KCl	0.66 ± 0.04	Contraction
0 Ca^{2+} + KCl	$0.16 \pm 0.03^{**}$	None
Phenylephrine	0.65 ± 0.05	Contraction
0 Ca^{2+} + phenylephrine	$0.06 \pm 0.04^{**}$	None
Wortmannin	$0.16 \pm 0.07^{**}$	Relaxation ($52.8 \pm 9.0\%$)
Wortmannin + KCl	$0.06 \pm 0.02^{**}$	None

Rabbit urethral smooth muscle strips were treated with KCl, phenylephrine or vehicle (see legend to Fig. 2) and the contractile responses were recorded. Tissues were immersed in TCA/acetone/DTT on wet ice, and LC_{20} phosphorylation was analysed by Phos-tag SDS-PAGE. Values indicate phosphorylation stoichiometry \pm SEM (2 tissue strips from each of 2 animals were analysed in each case). Statistically significant differences from control, as indicated by one-way ANOVA with Dunnett's post-hoc test, are indicated with asterisks ($^{**}p < 0.01$).

679FIGURE LEGENDS

680Fig. 1. Effects of ROK inhibition on urethral smooth muscle contraction. Rabbit urethral smooth muscle
681strips were dissected, mounted on a force transducer at resting tension and perfused with Krebs solution
682at 37 °C for at least 1 h. Two control contraction-relaxation cycles were recorded prior to incubation of
683the tissue with either H1152 (1 µM) or Y27632 (10 µM) for 15 min. Two contraction-relaxation cycles
684were recorded in the continued presence of inhibitor. The inhibitor was then washed out and two control
685contraction-relaxation cycles recorded again. *A*, representative traces depicting the effects of H1152 on
686contractions elicited by EFS (upper panel), KCl (middle panel) and phenylephrine (lower panel). *B*,
687cumulative data showing the effects of ROK inhibitors on contractions elicited by: electric field
688stimulation (EFS) at 4 Hz in the presence of H1152 ($n = 6$) or Y27632 ($n = 8$ with Y27632 and $n = 6$
689following washout), KCl (80 mM) stimulation in the presence of H1152 ($n = 5$ with H1152 and $n = 7$
690following washout) or Y27632 ($n = 4$), or phenylephrine (PHE; 10 µM) stimulation in the presence of
691H1152 ($n = 6$) or Y27632 ($n = 9$ with Y27632 and $n = 8$ following washout). Values indicate maximal
692tension as a percentage of the average tension of the initial control responses. Recovery (grey bars)
693indicates the average tension response following washout of the inhibitor (not significantly different
694from control). Statistically significant differences from control (absence of H1152), detected by one-
695way ANOVA with Dunnett's post-hoc test, are indicated by asterisks (** $p < 0.01$, $n = 5$). *C*, Effect of
696H1152 on sustained phenylephrine-induced contraction of urethral smooth muscle. Rabbit urethral
697smooth muscle strips equilibrated in Krebs solution at 37 °C were contracted with phenylephrine (10
698µM). Following washout and relaxation, a sustained contractile response was elicited with
699phenylephrine. H1152 (1 µM) was added in the continued presence of phenylephrine, following which
700both phenylephrine and H1152 were washed out and a final control sustained contraction elicited with
701phenylephrine.

702 Fig. 2. Effect of H1152 on phenylephrine-induced contraction of non-perfused rabbit urethral smooth
703 muscle strips at 21 °C. Rabbit urethral smooth muscle strips were dissected, mounted on a force
704 transducer at resting tension and equilibrated with Krebs solution at 21 °C for at least 1 h. *A*, Two
705 control phenylephrine (10 µM)-induced contraction-relaxation cycles were recorded prior to incubation
706 of the tissue with H1152 (1 µM) for 15 min. Two contraction-relaxation cycles were recorded in the
707 continued presence of inhibitor, which was then washed out and two control contraction-relaxation
708 cycles recorded again. *B*, Following two control phenylephrine-induced contraction-relaxation cycles,
709 phenylephrine was added again. The phenylephrine solution was changed 7 times and force was
710 maintained. Following washout, another contraction was elicited by phenylephrine and increasing
711 concentrations of H1152 (0.1, 0.25, 0.5, 1, 2.5, 5 and 10 µM) were applied in the continued presence of
712 phenylephrine, followed by washout of phenylephrine and H1152. Although not clearly apparent in this
713 figure, due to compression of the time scale, steady-state force was achieved before each addition of
714 H1152, as seen on the expanded time scale of the recorder. *C*, Cumulative data showing the effects of
715 increasing H1152 concentration on phenylephrine-induced contraction. In these experiments, longer
716 time intervals were used between successive additions of H1152 than in panel *B* to ensure that steady-
717 state force had been achieved.

718 Fig. 3. LC₂₀ phosphorylation in rabbit urethral smooth muscle treated with phenylephrine or KCl, and
719 the effect of ROK inhibition. Rabbit urethral smooth muscle strips were dissected, mounted on a force
720 transducer at resting tension and equilibrated with Krebs solution at 21 °C for at least 1 h prior to
721 treatment with phenylephrine (10 µM), KCl (80 mM), calyculin-A (5 µM) or vehicle (control). Tissues
722 were quenched in TCA/DTT in acetone on wet ice once steady-state force was developed, washed with
723 DTT in acetone and lyophilized overnight before extraction of tissue proteins with SDS-gel sample
724 buffer. Phosphorylated and unphosphorylated forms of the 20 kDa myosin regulatory light chains (LC₂₀)

were separated by SDS-PAGE (in duplicate) with polyacrylamide-bound Mn^{2+} -phosphate-binding tag (Phos-tag SDS-PAGE) and detected by western blotting with anti-LC₂₀ (A) or phosphospecific anti-LC₂₀, which recognizes only LC₂₀ phosphorylated at S19 (B). "LC₂₀" denotes unphosphorylated LC₂₀, "P₁-LC₂₀" denotes LC₂₀ phosphorylated at S19 and "P₂-LC₂₀" denotes LC₂₀ phosphorylated at S19 and T18. The position of the 28 kDa marker is indicated at the right. C, Triplicate samples of muscle strips treated with KCl (80 mM) or phenylephrine (10 μ M) were subjected to Phos-tag SDS-PAGE in the presence of MnCl₂ (upper panel) or EDTA (lower panel). The position of the 26 kDa marker is indicated at the right. D, Rabbit urethral smooth muscle strips were pre-incubated with the ROK inhibitor H1152 (1 μ M) prior to addition of phenylephrine (10 μ M), KCl (80 mM) or vehicle. LC₂₀ phosphorylation was analysed by Phos-tag SDS-PAGE. The position of the 28 kDa marker is indicated at the right.

Fig. 4. Time courses of LC₂₀ phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine. Typical KCl (80 mM)- (A) and phenylephrine (10 μ M)-induced (B) contractile responses. Numbers indicate the times during contractions at which tissues were quenched for Phos-tag SDS-PAGE analysis of LC₂₀ phosphorylation (C). D, Cumulative data (values indicate the mean \pm SEM, $n = 3$ for KCl and $n = 2$ for phenylephrine).

Fig. 5. MYPT1 phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine. Rabbit urethral smooth muscle strips were treated with phenylephrine (10 μ M), KCl (80 mM) or vehicle (control) and the phosphorylation of MYPT1 at the peak of the contractile response was analysed in triplicate by western blotting with phosphospecific antibodies that recognize MYPT1 phosphorylated at T697 or T855. Loading levels were normalized to the actin-binding protein calponin.

Fig. 6. Time courses of MYPT1 phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine. Rabbit urethral smooth muscle strips were treated with 80 mM KCl (A) or 10 μ M phenylephrine (B) and the phosphorylation of MYPT1 was analysed in triplicate at selected times during

748the contractile response by western blotting with phosphospecific antibodies that recognize MYPT1
749phosphorylated at T697 or T855. Loading levels were normalized to actin. Numbers beneath gel lanes
750indicate the times during contractions at which tissues were quenched for western analysis of MYPT1
751phosphorylation (see Fig. 4A and B). C, Cumulative data (values indicate the mean \pm SEM, $n = 4$ for
752KCl and $n = 3$ for phenylephrine).

753Fig. 7. Phosphatase inhibition with microcystin markedly increases MYPT1 phosphorylation at T697
754and T855. Rabbit urethral smooth muscle strips (intact or Triton-skinned) were treated with KCl (80
755mM, intact strips), microcystin (10 μ M, Triton-skinned strips) or vehicle (control, intact strips) and the
756phosphorylation of MYPT1 (130 kDa) analysed in triplicate by western blotting with phosphospecific
757antibodies. Loading levels were normalized to calponin (32 kDa). "M" denotes the molecular weight
758marker lane.

759Fig. 8. Cofilin phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine.
760Rabbit urethral smooth muscle strips were treated with 80 mM KCl (A) or 10 μ M phenylephrine (C) and
761the phosphorylation of cofilin was analysed at selected times during the contractile response by western
762blotting with phosphospecific antibodies that recognize cofilin phosphorylated at S3. Loading levels
763were normalized to LC₂₀ and calponin. Numbers beneath gel lanes indicate the times during contractions
764at which tissues were quenched for analysis of cofilin phosphorylation (see Fig. 4A and B). B and D,
765Cumulative data for KCl- and phenylephrine-induced contractions, respectively (values indicate the
766mean \pm SEM, $n = 4$ (B), $n = 3$ (D)).

767Fig. 9. Analysis of actin polymerization during KCl- and phenylephrine-induced contraction of rabbit
768urethra. Rabbit urethral smooth muscle strips were treated with KCl (80 mM) or phenylephrine (10 μ M).
769Tissues were homogenized, centrifuged at low speed to remove cell debris and then at high speed to
770separate F- and G-actin. The high-speed supernatants and pellets were analysed by western blotting with

771 anti-actin and anti-SM-22, and representative results are shown in *A*: lanes 1, 3, 6 and 8: KCl-treated
772 tissues (15, 7.5, 7.5 and 15 μ l, respectively); lanes 2, 4, 5 and 7: phenylephrine-treated tissues (15, 7.5,
773 7.5 and 15 μ l, respectively). *B*, Tissues were homogenized at selected times during the contractions and
774 centrifuged at high speed to remove F-actin. The high-speed supernatants were analysed by western
775 blotting with anti-actin and anti-SM-22. Time courses in response to KCl or phenylephrine treatment are
776 shown. Numbers beneath gel lanes in (*B*) indicate the times during contractions at which tissues were
777 homogenized for western analysis of actin and SM-22 (see Fig. 4*A* and *B*).

FIGURE 1A

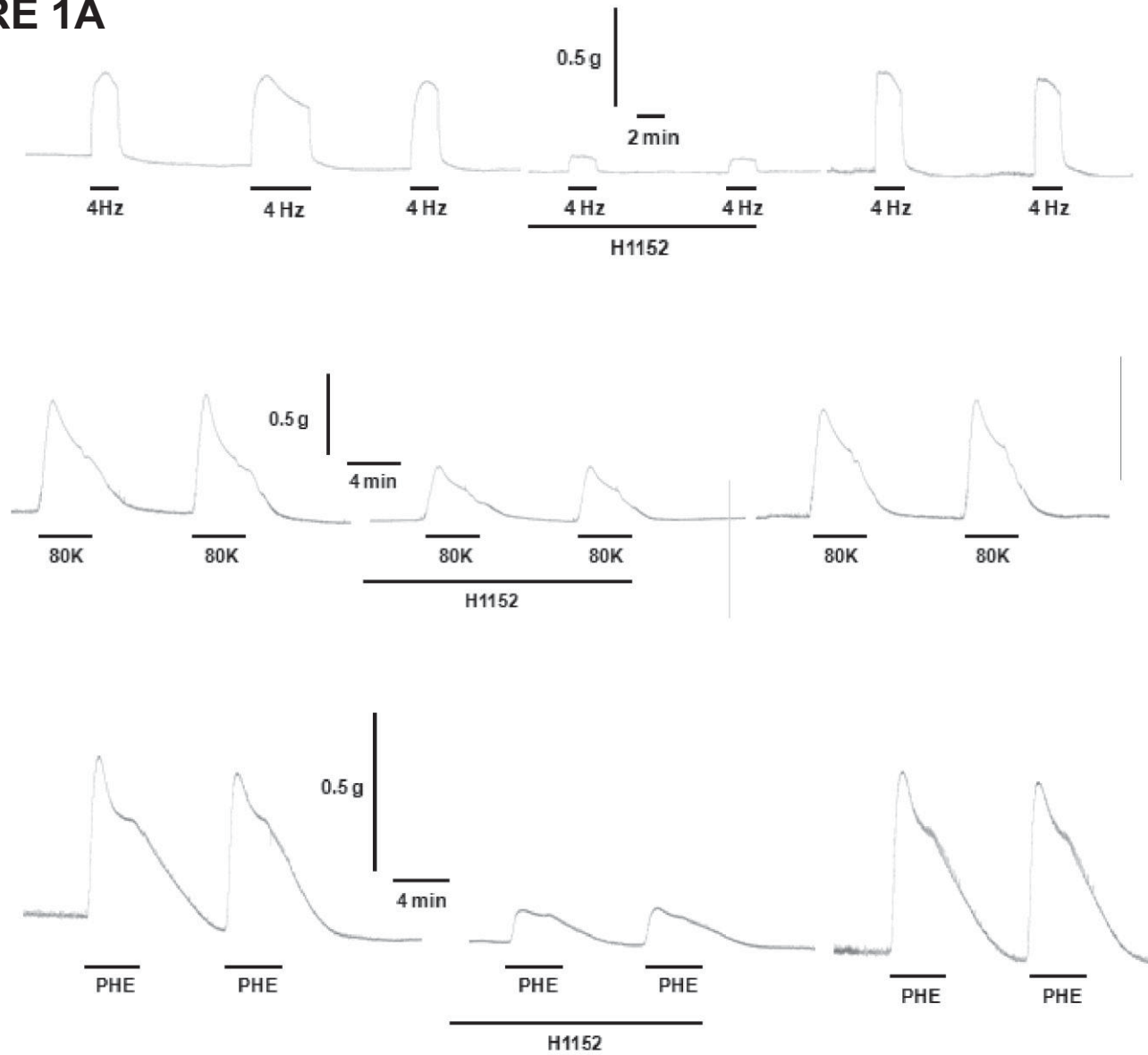


FIGURE 1B

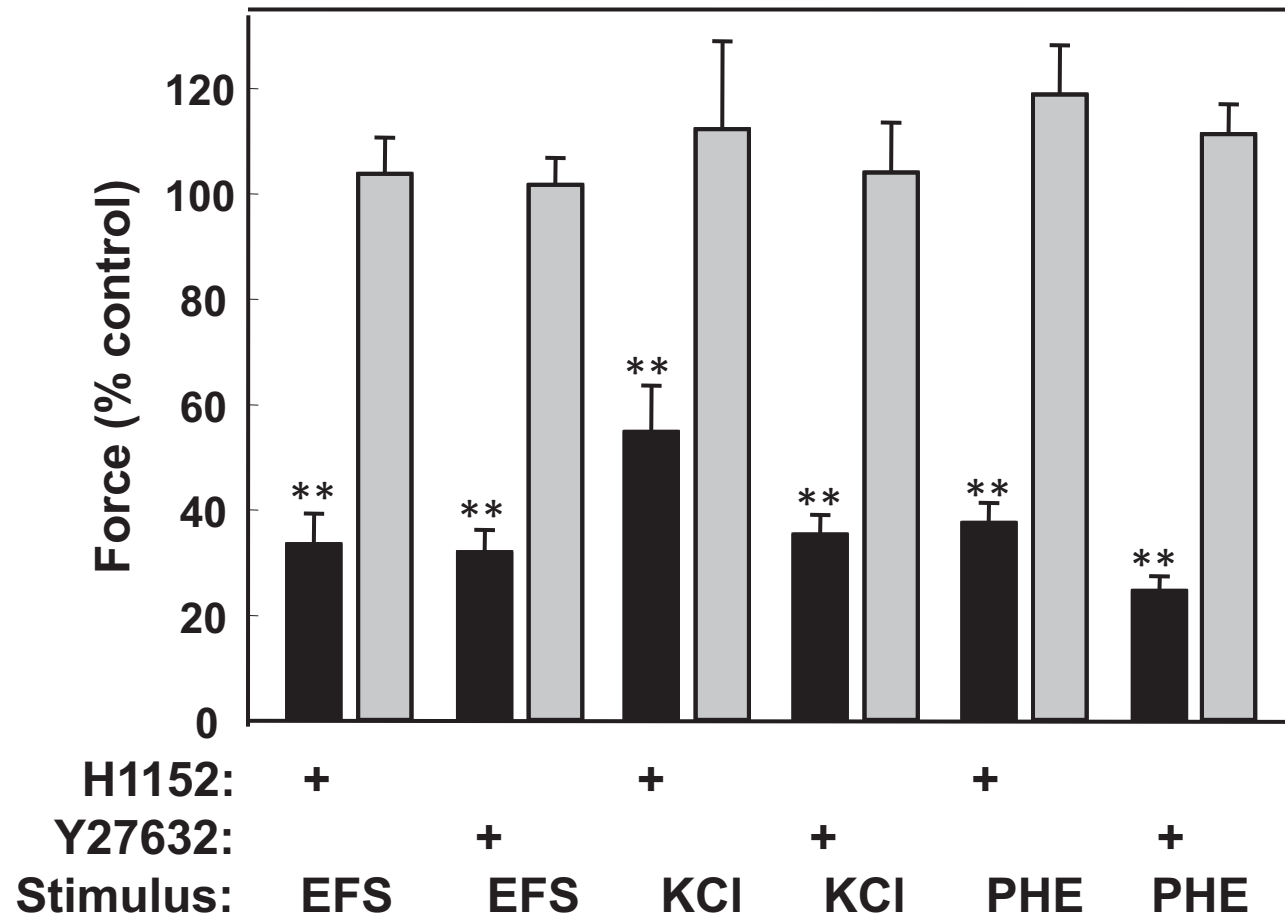


FIGURE 1C

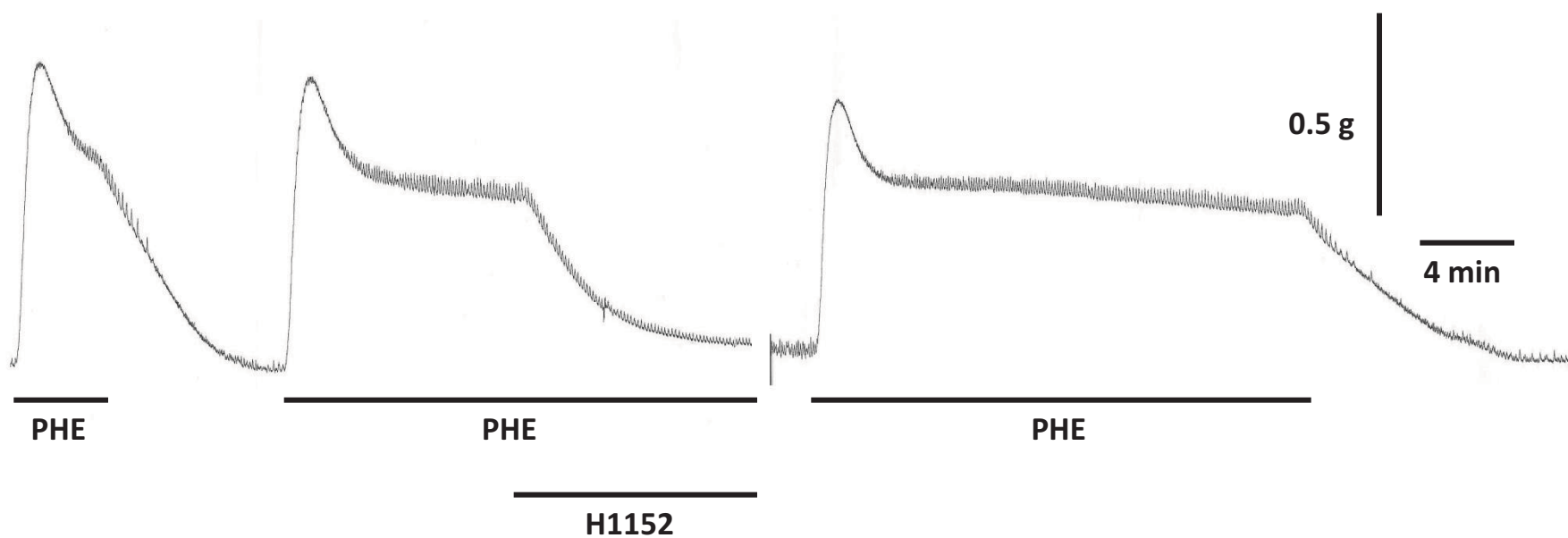


FIGURE 2A

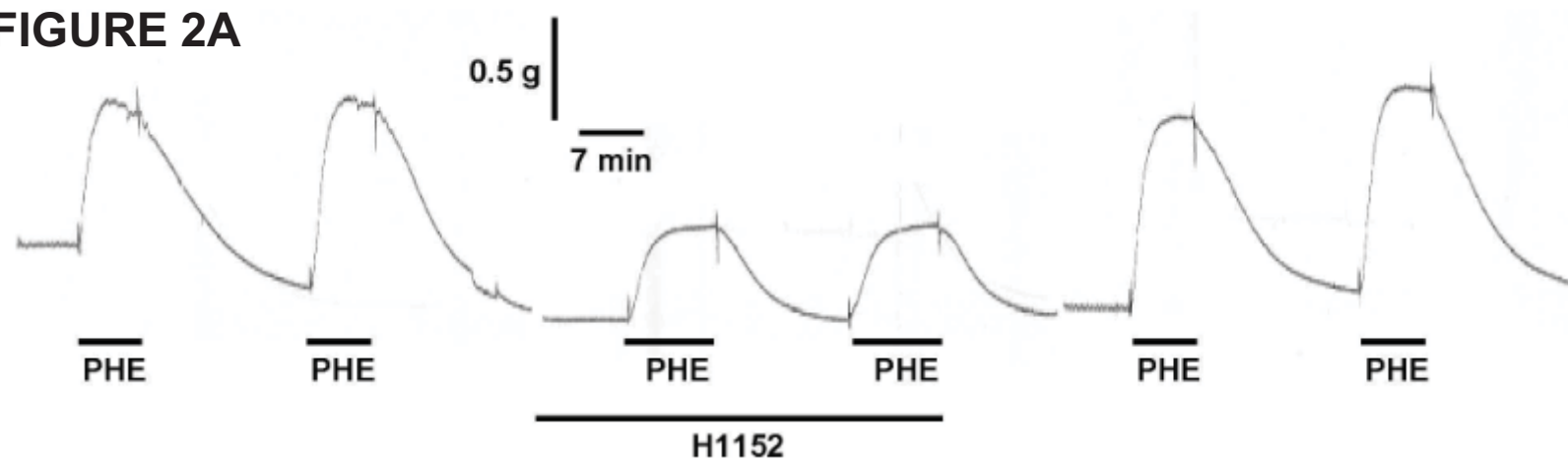


FIGURE 2B

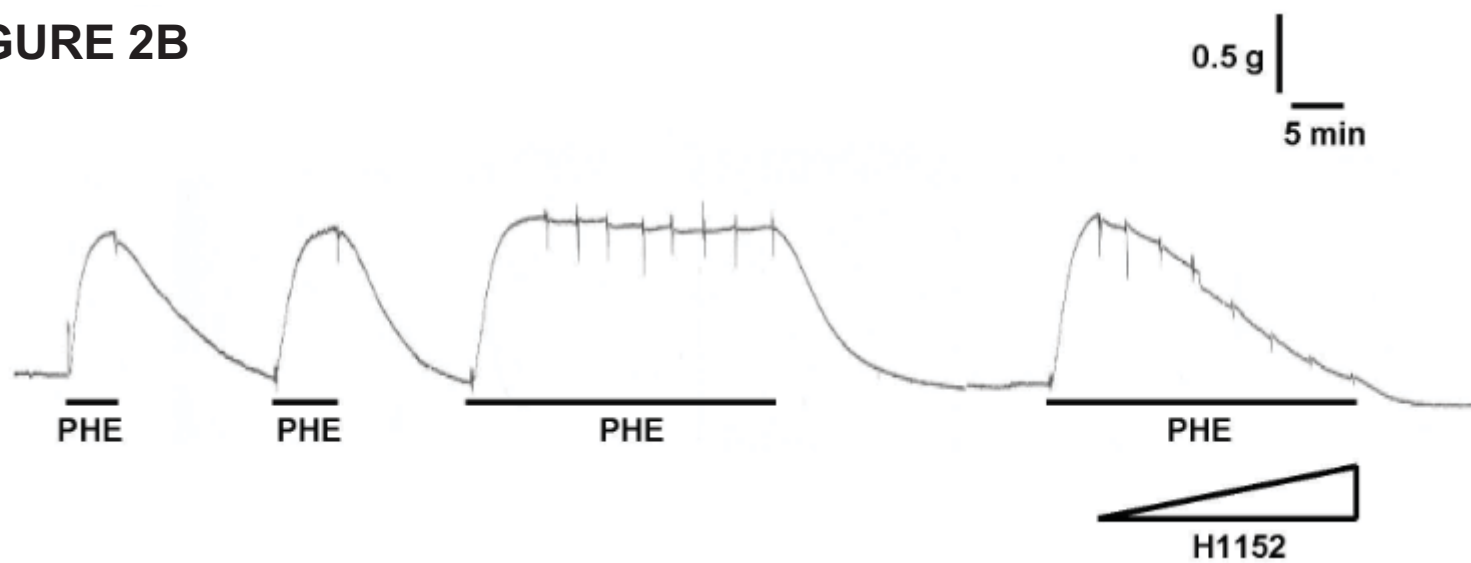


FIGURE 2C

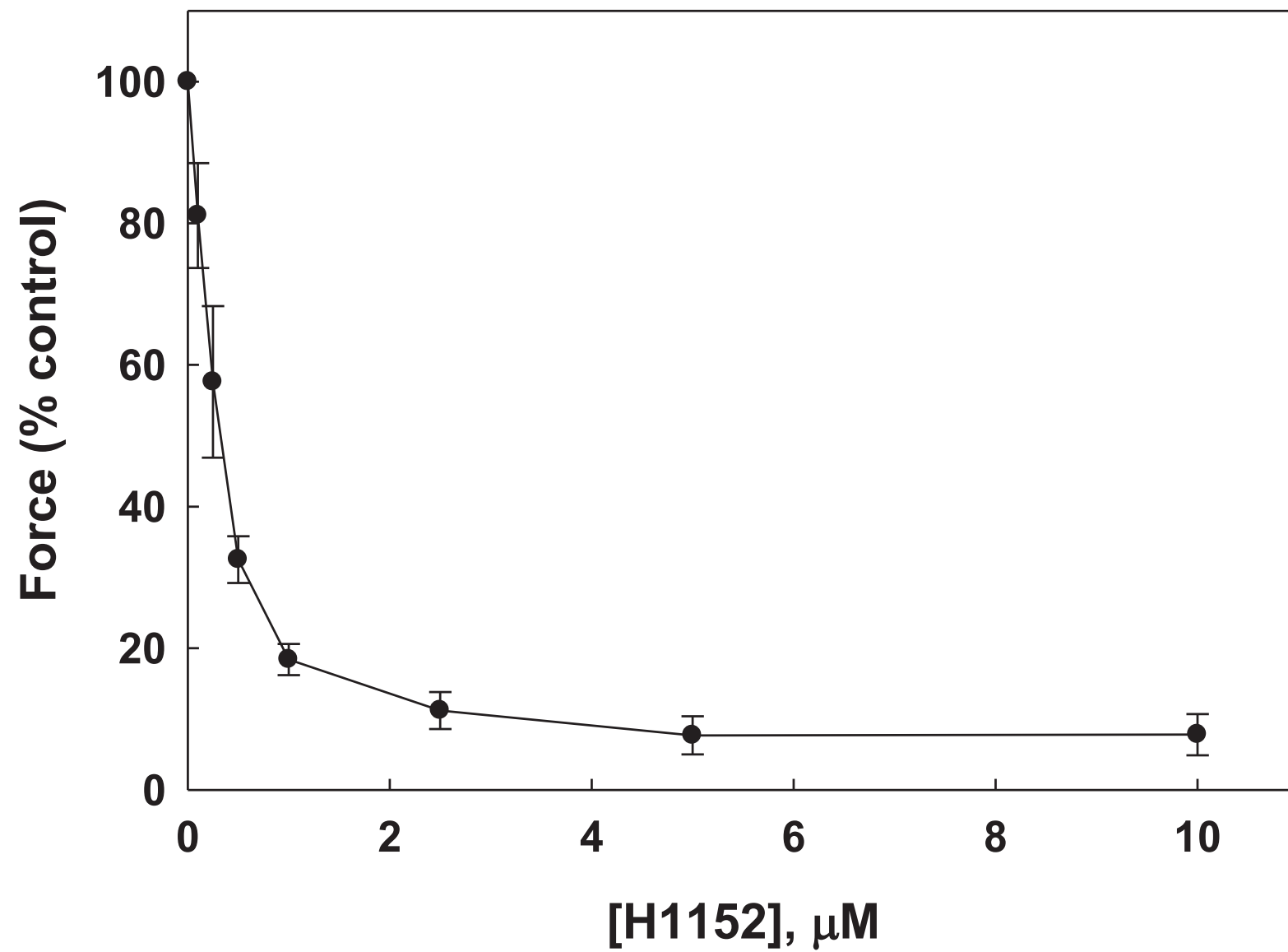


FIGURE 3

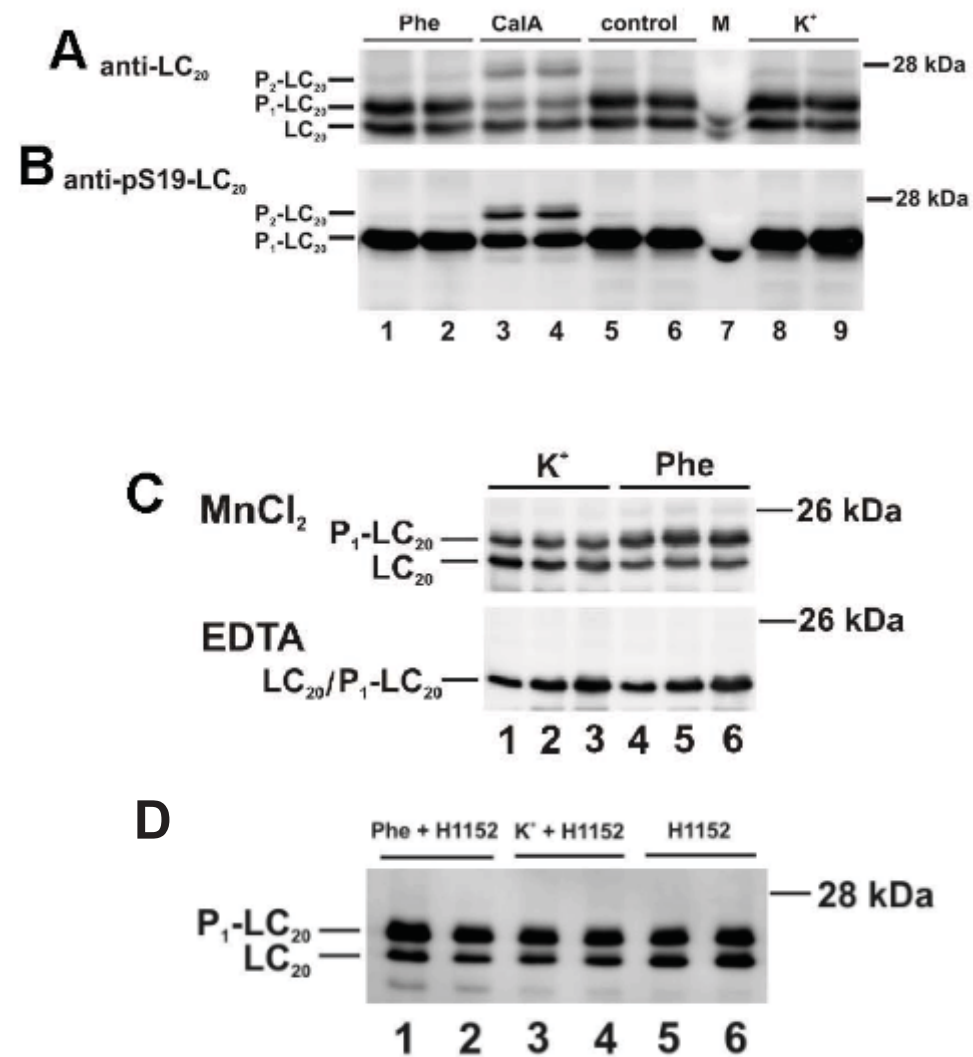


FIGURE 4

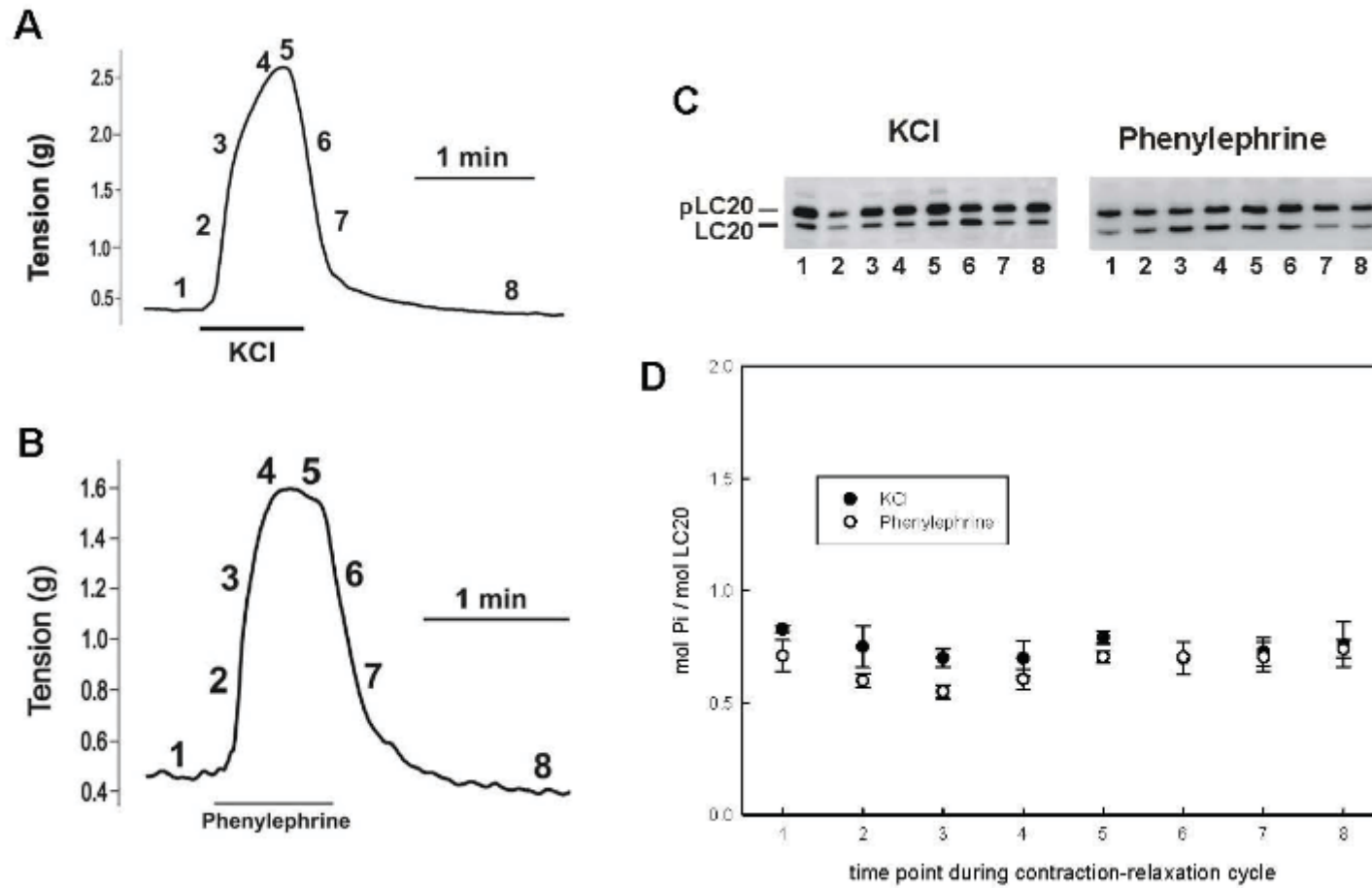


FIGURE 5

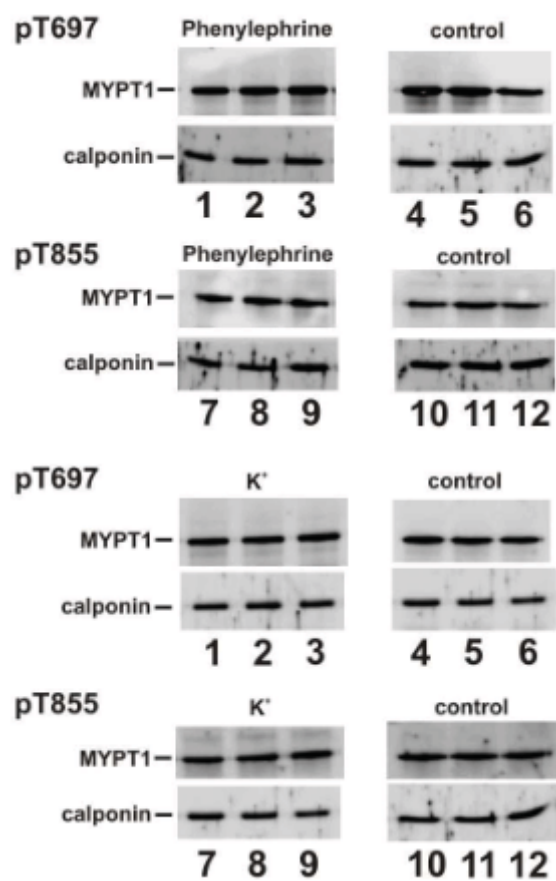


FIGURE 6

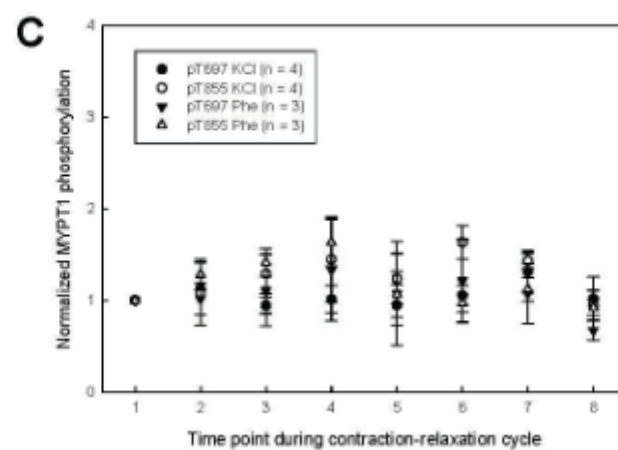
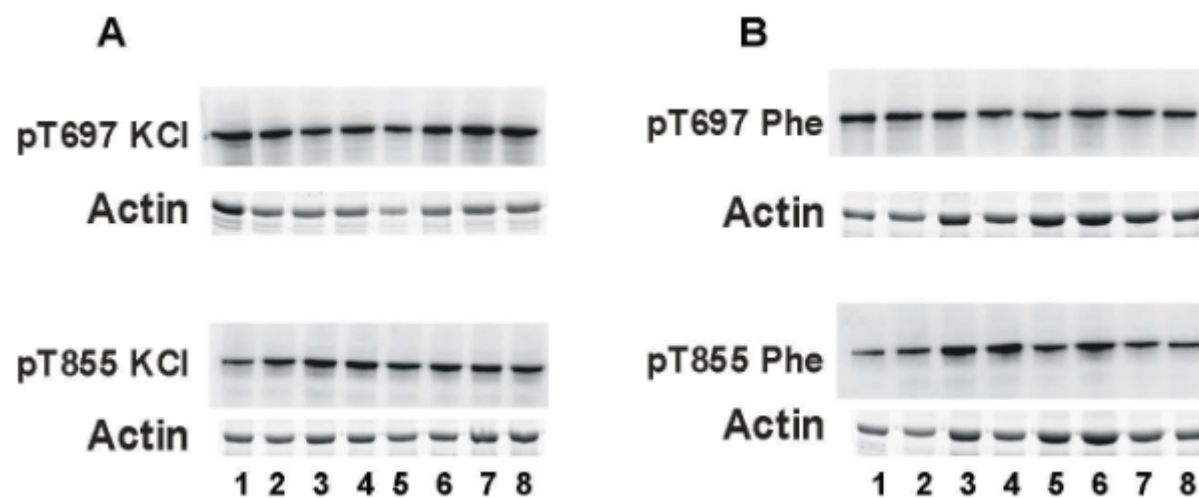


FIGURE 7

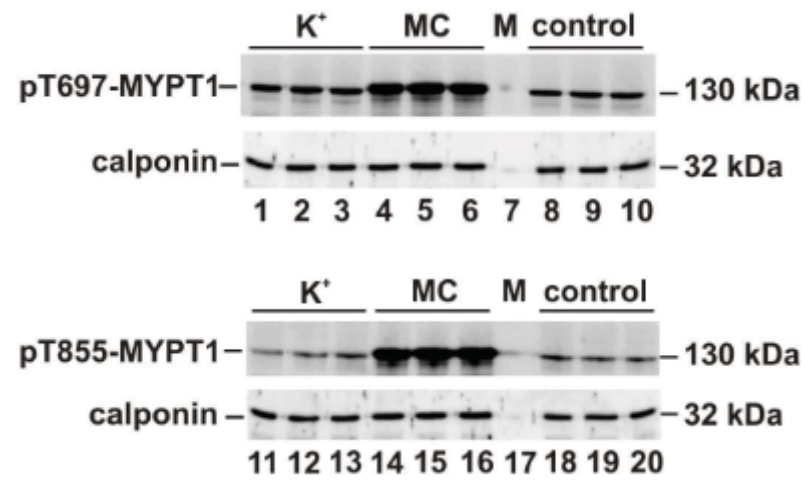
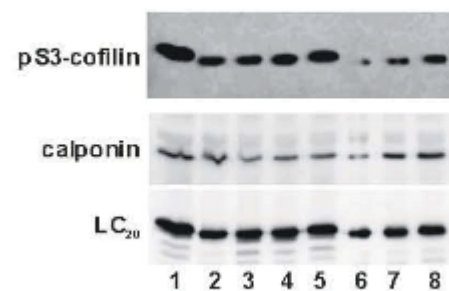
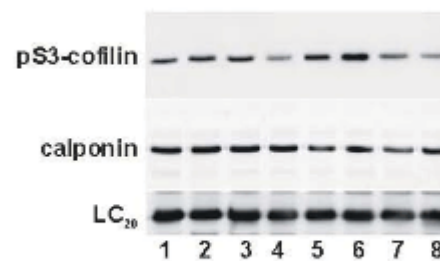


FIGURE 8

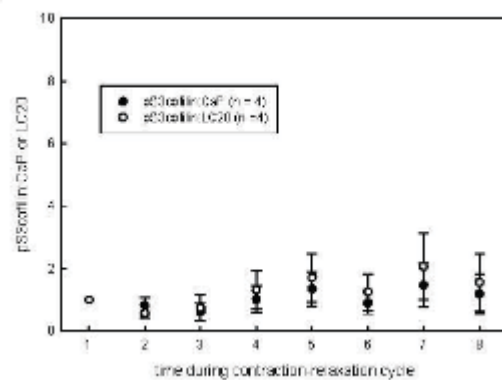
A



C



B



D

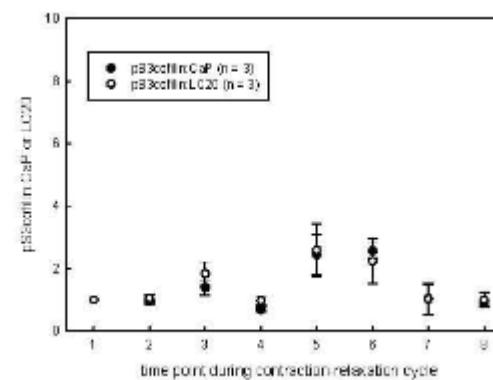


FIGURE 9

